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THE EFFECT OF FEEDING NEUTRALIZER ON THE GROWTH OF *BIFIDOBACTERIUM BIFIDUM*

Guowei SHU*[1], Shuai WANG*, He CHEN*, Man HU*, Tao QIN**, Qi MA**

**School of Food and biological Engineering, Shaanxi University of Science and Technology, Xi' an, China*
*** Enzyme Engineering Institute, Shaanxi Academy of Sciences, Xi'an, China*

Abstract: In order to investigative the effect of different neutralizers and their feeding time on culture of *Bifidobacterium bifidum BB01*, and pH, OD and viable count of *B. bifidumBB01* in the medium in different time were measured. The results indicated that the NaOH solution was the optimum neutralizer compared with the others, and feeding time of the neutralizer to *B. bifidum* BB01 was 13 hours after inoculation. Furthermore, the OD value and viable count reached maximum at 21h (OD value= 1.667) and 20h (viable count: (3.52±0.046) $\times 10^9$ CFU/mL) after the NaOH solution was added to the medium, respectively. In addition, the maximum OD value implied that the logarithmic phase of *B. bifidum BB01* was delayed compared with the control and the viable count were 29.26% larger than the control group. The result of the study provides a method and an important basis for improving the viable counts of *B. bifidum BB01*.

Keywords: probiotics, *Bifidobacterium bifidum*, neutralizers feeding, proliferation

INTRODUCTION

Bifidobacteria have raised attention due to their capability to adjust the immune response (Schultz, et al., 2004; Vissers et al., 2010) through their effects on human intestinal microflora. They are considered to have many beneficial effects on the host organism, including prevention and treatment of intestinal disorders, serum cholesterol reduction, stimulation of immune system, anti-carcinogenic activity, anti-mutagenic, produce antibiotics (Yusof, et al., 2000), improve the vitamin and protein metabolism, treating liver damage, relieve lactose intolerance and other functions (Arunachalam, 1999). Bifidobacteria are widely applied in fermented dairy products and probiotic adjuncts, such as yogurts and fermented milks (Ross et al., 2005), Sanchez et al., 2009).

[1] Corresponding author. Mailing address: Guowei Shu, 37# School of Food and biological Engineering, Shaanxi University of Science & Technology, Weiyang district, Xi'an city, Shaanxi Province, China. E-mail:shuguowei@gmail.com

It is well known that the viable counts of the probiotic bacteria are the important factors on efficacy, transportation and storage, moreover, the growth of bacterial cultures vary depending on the growth medium and growth condition (Dietrich Knorr, 1998). On the one hand, it's well documented that the addition of sucrose (Costa et al., 2000; Palmfeldt et al., 2003), oligosaccharides (Falony et al., 2009), the plant cell wall polysaccharide arabinogalactan (Degnan and Macfarlane, 1995), fructose and inulin (Ozer and Akin, 2005) in media could increase the concentration of bifidobacteria in medium (Tao et al., 2007). Bifidobacteria were growing well on pure carrot juice without nutrient supplementation, the cell concentrations from 107 CFU/mL to 108 CFU/mL after 6h of incubation (Szilard Kun et al., 2008). Janer et al. reported that caseinomacropeptide could be fed to complement milk in order to increase the number of bifidobacteria in probiotic fermented milks (Janer et al., 2004).

On the other hand, bfidobacteria fermentation accumulated acetic and lactic acids cause media acidification, which inhibit growth of this bacterium, because bifidobacteria are less acid-tolerant (Kailasapathy and Chin, 2000), (Gomes et al., 1999). The optimum pH value for growth of bifidobacteria is 6.5-7.0, and it inhibit growth when pH value below 5.0 or above 8.0, furthermore, the acidic environment ($pH \leq 5.5$) is not suitable for cell growth (Du et al., 2009). Therefore, adding neutralizing agent to keep suitable acidity can make the viable count achieves a higher level.

In previous work, effects of ascorbic acid, cysteine hydrochloride, stachyose, xylooligosaccharide, galatooligosaccharide on growth of *Bifidobacterium bifidum* BB01 were studied (Shu et al., 2011),(Shu et al., 2013). The aims of the present work were to investigate three neutralizers (NaOH, ammonia, Na_2CO_3) and feeding time, which can encourage growth of *Bifidobacterium bifidum* BB01. The results will be helpful to provide a method for obtaining higher viable counts.

MATERIALS AND METHODS

Microorganism: The probiotic strains employed in the present study was *Bifidobacterium bifidum* BB01, which were obtained from School of Food and biological Engineering, Shaanxi University of Science and Technology.

Activation and cultivation method: *Bifidobacterium bifidum* BB01 freeze-dried powder were inoculated to MRS broth (Hope Bio-technology CO. LTD, Qingdao, China), and cultured 37 ◦C for 24h. Then 5% active culture was inoculated into the medium and incubated at 37 ◦C for 18h until the viability of bacteria stays stable and the OD value reached a certain requirements.

Determination of pH: The pH of culture media was measured by a pH meter (pHS-3C Shanghai Precision Scientific Instrument Co., Ltd, Shanghai, China).

Determination of OD value: The optical density at 600nm (OD600) was monitored through a spectrophotometer (SP-756PC, Shanghai Spectrum Co., Ltd., Shanghai, China).

Determination of bacterial-growth curve: 5% active culture was inoculated into MRS broth. The growth media was incubated at 37◦C and sampled every two hours to measure the OD value, pH value and viable count, respectively. Draw the growth curve based on the experimental data.
Determination of viable count: Through a serial the gradient dilution on sterile saline solution, and used a syringe with 0.1mL diluted bacterial suspension dropped into a count plate and then measured the viable counts.
The number of viable cells was determined by the spread plate technique using MRS agar (triplicate for each sample). Plates were anaerobically incubated at 37 ◦C for 48 h, and the viable counts were represented by CFU/mL.
Cultivation of feeding test: Feeding time of the neutralizer and the influence of addition of neutralizers on pH and OD were studied. Preliminary determined the loading time of neutralizer, then determined the optimum neutralizing agent by studying the effect of different neutralizers on the viable counts.

RESULTS AND DISCUSSIONS

The determination of feeding point time and optimal neutralizing agent

The OD value and pH value of the medium were measured every one hour. The results were shown as Table 1.

Table 1. The changes of OD and pH level by cultivation of *Bifidobacterium bifidum*

culture time(h)	OD	pH
3	0.211	5.25
4	0.345	5.17
5	0.550	5.02
6	0.808	4.85
7	0.953	4.68

The results showed that the pH of culture medium was gradually decreased and the OD value gradually increased in incubation time. The pH value closed to 5.0 at 5h and in the sixth hour pH below 5.0. Due to the growth of bifidobacteria in pH below 5.0 become slowly (Du et al., 2009), the cell death occurred after 5 hours. Therefore, the point time at 5h for adding neutralizing agent was chosen.
Furthermore, the selected neutralizing agent (1 mol/L NaOH solution, 1 mol/L $NaCO_3$ solution and 2 mol/L ammonia) were added to MRS broth after 5h, respectively. Then the OD value, viable counts and pH value of the medium were measured at 9h, 12h, 15h, 18h, respectively (Figure 1). The pH value of each group decreased rapidly after 9h, it's showed that the neutralizer neutralized bacteria produced acid in the growth process. The viable counts were increase after feeding the neutralizer. Viable counts of the experimental group of adding Na_2CO_3 were increase gradually in 9-12h, and declined after 12h, that might be the CO_2 produced by Na_2CO_3 could not be ruled out and dissolved in the medium decreased the acidity in the culture medium.

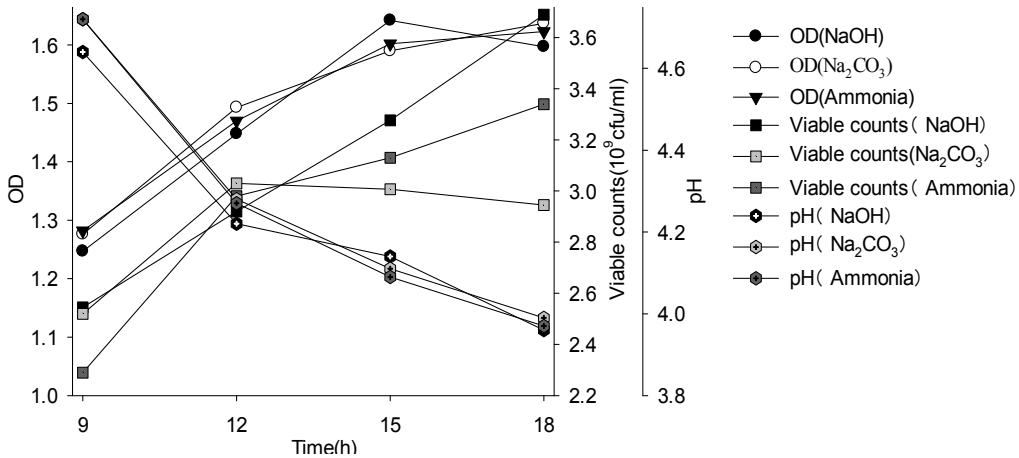

Figure 1. The effect of feeding alkali in medium on *Bifidobacterium bifidum BB01*. OD, pH and viable counts.

Viable counts of the experimental group added ammonia were increase gradually in 9-12h, but increased more slowly after 12h. Viable counts of the experimental group adding NaOH were rapidly increased than the other two groups in 9-18h. This indicated that the neutralizer removed the influence of acid on the growth inhibition, especially NaOH. The viable counts of the three experimental groups reached a maximum 3.69×10^9 CFU/mL, 3.03×10^9 CFU/mL, 3.34×10^9 CFU/mL at 18h, 12h, 18h, respectively. And the highest viable counts demonstrated that NaOH is the best neutralizing agent of *Bifidobacterium bifidum* BB01.

Determination of feeding time

The OD value, pH value and viable counts of the medium were measured during every 2h to investigate the growth curve of *Bifidobacterium bifidum* BB01 (Figure 2). The pH value of the medium was reduced, which indicated that the proliferation of microorganisms produced a large amount of acidic substances. *Bifidobacterium bifidum* entered into logarithmic growth period at 4h and reached the late logarithmic growth period at 13h. Number of viable cells began to decline after 18h. This showed that the proliferation and metabolism of Bifidobacteria and metabolic acid causes the reduction in pH, which further inhibited the growth of bifidobacteria. Therefore, the late logarithmic growth phase (at 13h) was selected as the optimal feeding point time that added NaOH to neutralize the acid and prolong the logarithmic growth phase.

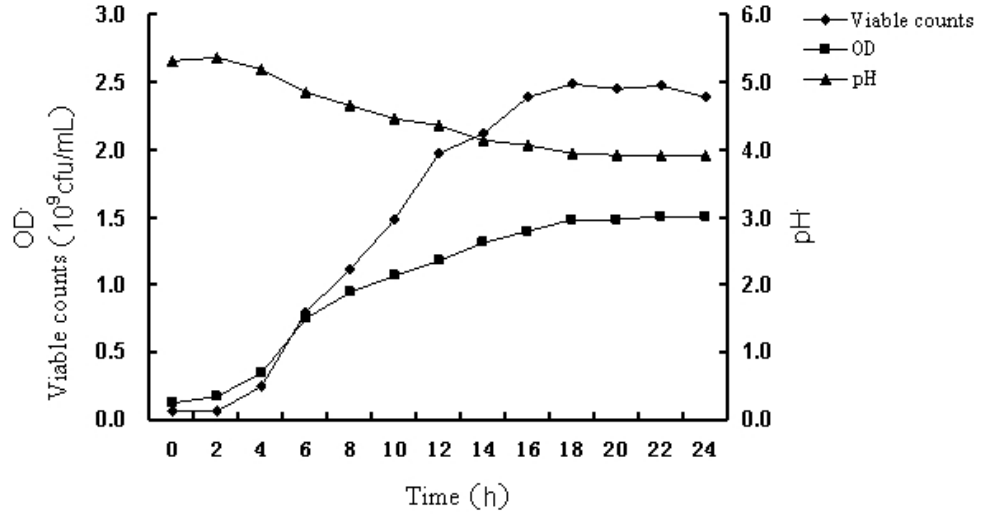

Figure 2. The effect of feeding alkali in medium on *Bifidobacterium bifidum*.OD, pH and viable counts.

Effect of NaOH on the growth of microorganism

Feeding (feeding NaOH at 13h) and control groups (without NaOH) were compared by cultured under identical conditions. NaOH were added with a certain volume of 1mol/L to adjust the liquid pH value to 6.5 when *Bifidobacterium bifidum* BB01 were cultured to 13h, and then cultured to 24h. The OD value, pH value and viable count measured every 1h after adding alkali, the results shown in Figure 3.

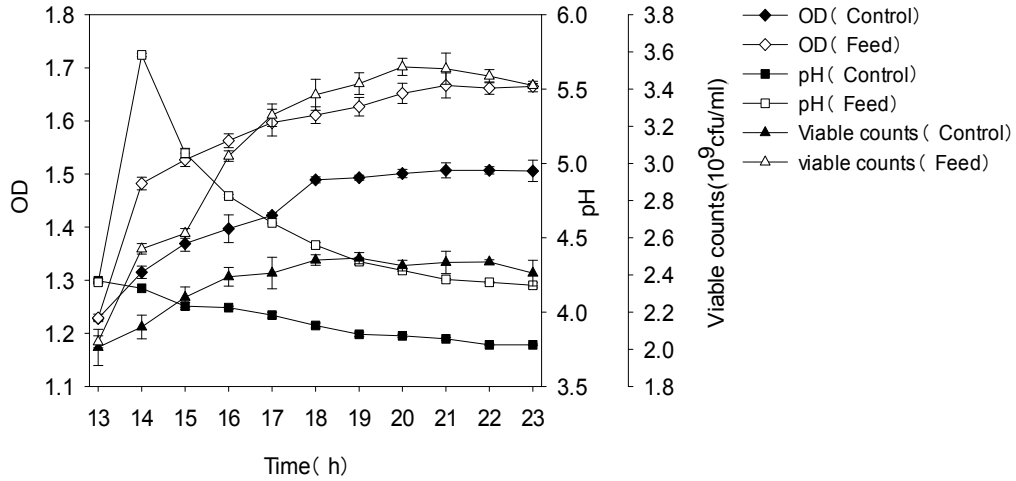

Figure 3. The result of verification test on feeding NaOH, OD, pH and viable counts.

The viable counts (control group) increased slowly after 13h, this was owing to accumulation of acid that inhibit the growth of *Bifidobacterium bifidum* BB01. On

the contrary, after addition of NaOH at 13h, the pH value was higher and the viable counts increase again. In addition, viable counts of feeding group reached maximum at 20h (3.52±0.046)×10^9CFU/mL, a 29.26% increase than the control group (18h (2.49±0.03)×10^9 CFU/mL). The OD value of feeding group reached 1.667 at 21h can also suggest this. Compared with the control group, the time of cell logarithmic growth period was prolonged and reproductive capacity was enhanced. The pH value of the medium dropped to below 4.20 reached the late logarithmic growth after 21h, further proof that the logarithmic feeding could prolong the microbial growth and promoted cell growth. It was proved that the adding alkali extended the microbial logarithmic growth phase and *bifidobactera* enrichment effect was remarkable, viable counts in medium had a larger increase than before.

CONCLUSIONS

Addition of NaOH in MRS media could improve the buffer ability of MRS media and have the significant promotion on growth of *Bifidobacterium bifidum*. The effect of NaOH solution on bacterial growth was significantly, and the viable counts in medium reached the highest (3.52±0.046) ×10^9 CFU/mL at 20h when incubation, a 29.26% increase than the control group (18h (2.49±0.03) ×10^9 CFU/mL) was reached. In conclusion, addition of NaOH played an important role in promoting growth of *Bifidobacterium bifid*um and provided reference for obtaining higher viable counts.

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